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Modulation of Cellular Calcium by Sigma-2 Receptors: Release from Intracellular Stores in Human SK-N-SH Neuroblastoma Cells

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ABSTRACT

Human SK-N-SH neuroblastoma cells expressed sigma-1 and sigma-2 receptors with similar pharmacological profiles to those of rodent-derived tissues, although sigma-2 receptors exhibited some affinity differences that might suggest heterogeneity or species differences. Structurally diverse sigma ligands produced two types of increases in intracellular (cytosolic) Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in these cells. CB-64D, CB-64L, JL-II-147, BD737, LR172, BD1008, haloperidol, reduced haloperidol, and ibogaine all produced an immediate, dose-dependent, and transient rise in $[\text{Ca}^{2+}]_i$. Sigma-inactive compounds structurally similar to the most active sigma ligands and ligands for several neurotransmitter receptors produced little or no effect. The high activity of CB-64D and ibogaine (sigma-2-selective ligands) compared with the low activity of (+)-pentazocine and other (+)-benzomorphans (sigma-1-selective ligands), in addition to enantioselectivity for CB-64D over CB-

64L, strongly indicated mediation by sigma-2 receptors. The effect of CB-64D and BD737 was blocked by the sigma antagonists BD1047 and BD1063, further confirming specificity as a receptor-mediated event. The transient rise in $[\text{Ca}^{2+}]_i$ occurred in the absence of extracellular Ca^{2+} and was completely eliminated by pretreatment of cells with thapsigargin. Thus, sigma-2 receptors stimulate a transient release of Ca^{2+} from the endoplasmic reticulum. Prolonged exposure of cells to sigma-receptor ligands resulted in a latent and sustained rise in $[\text{Ca}^{2+}]_i$, with a pharmacological profile identical to that of the transient rise. This sustained rise in $[\text{Ca}^{2+}]_i$ was affected by neither the removal of extracellular Ca^{2+} nor thapsigargin pretreatment, suggesting latent sigma-2 receptor-induced release from thapsigargin-insensitive intracellular Ca^{2+} stores. Sigma-2 receptors may use Ca^{2+} signals in producing cellular effects.

Sigma receptors are unique drug binding sites with pharmacological profiles and potential functions that are unrelated to other known receptors (for reviews, see Walker et al., 1990; Itzhak, 1994). Two pharmacologically defined subclasses of sigma-receptor are now widely accepted, termed sigma-1 and sigma-2 (Hellewell and Bowen, 1990; Quirion et al., 1992). Although both subtypes bind haloperidol and 1,3-di-*o*-tolylguanidine (DTG) with high affinity, sigma-1 receptors bind (+)-benzomorphans and (+)-morphinans with high affinity, whereas sigma-2 receptors exhibit low to negligible affinity for these compounds (Hellewell and Bowen, 1990; Quirion et al., 1992).

Sigma ligands have been found to produce a wide array of effects, implicating sigma-receptors in various biochemical, physiological, and behavioral processes. Notable among these are several modulatory actions demonstrated *in vitro*,

including the modulation of basal neurotransmitter synthesis or release, modulation of *N*-methyl-D-aspartate (NMDA)-glutamate receptor-stimulated neuronal activity, modulation of NMDA-glutamate receptor-stimulated dopamine and norepinephrine release, modulation of muscarinic receptor-stimulated phosphoinositide turnover, and modulation of potassium channel conductances (Walker et al., 1990; cf. Itzhak, 1994). Some behavioral effects of sigma-receptors include the regulation of movement and posture and involvement in learning and memory processes (Walker et al., 1990; cf. Itzhak, 1994). Sequence similarity and pharmacological overlap of the cloned sigma-1 receptor with the fungal sterol isomerase enzyme have suggested a role in sterol biosynthesis (Hanner et al., 1996). However, sigma-1 receptor protein lacks any enzymatic activity, and a clear role in sterol metabolism has yet to be demonstrated.

Little is known of the signal transduction mechanisms occurring at the cellular level that might underlie functional

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ABBREVIATIONS: DTG, 1,3-di-*o*-tolylguanidine; $[\text{Ca}^{2+}]_i$, intracellular (cytosolic) Ca^{2+} concentration; DMEM, Dulbecco's modified Eagle's medium; SERCA, sarcoplasmic-endoplasmic reticulum Ca^{2+} -ATPase; DPBS, Dulbecco's phosphate-buffered saline; IP₃, inositol-1,4,5-trisphosphate; Red HAL, reduced haloperidol; DAMGO, [D-Ala²,N-Me-Phe⁴,Gly-ol⁵]-enkephalin; NMDA, *N*-methyl-D-aspartate.

effects of sigma receptors. Ca^{2+} serves as an important intracellular signal for cellular processes such as growth and differentiation, regulation of gene expression, and cellular stimulus-secretion coupling (Clapham, 1995; Simpson et al., 1995; Berridge et al., 1998). Ca^{2+} is also known to be toxic to cells and is involved in the triggering of events leading to excitotoxic cell death in neurons, as well as apoptosis in various cell types (McConkey and Orrenius, 1996; Berridge et al., 1998). Maintenance of cellular Ca^{2+} homeostasis involves a coordinated control of several processes that change the intracellular Ca^{2+} concentration in the cytosol ($[\text{Ca}^{2+}]_i$). $[\text{Ca}^{2+}]_i$ can be altered by entry of Ca^{2+} from outside of the cell through 1) voltage-dependent channels, 2) voltage-independent channels or pores, and 3) ligand-gated Ca^{2+} channels. $[\text{Ca}^{2+}]_i$ can also be altered by changing the activity of the energy-dependent mechanisms that pump Ca^{2+} out of the cell (plasma membrane Ca^{2+} -ATPases) or those that concentrate Ca^{2+} from the cytoplasm into the endoplasmic reticular, sarcoplasmic reticular, and mitochondrial intracellular storage pools. Ca^{2+} can be released from intracellular storage pools by certain signals such as electrical stimulation in the case of the sarcoplasmic reticulum and by activation of inositol-1,4,5-trisphosphate (IP_3) receptors in the case of endoplasmic reticulum. The latter is the result of activation of G protein-coupled hormone or neurotransmitter receptors that are linked to activation of phospholipase-C and production of inositol phosphates and diacylglycerol. Mitochondria and other intracellular organelles can also be involved in Ca^{2+} signaling by playing a role in cytoplasmic Ca^{2+} buffering, thus helping to maintain Ca^{2+} homeostasis (Pozzan et al., 1994; Clapham, 1995; Simpson et al., 1995).

We have previously shown that sigma receptors mediate morphological changes in neuronal and non-neuronal cells and ultimately produce cell death on continued exposure in culture (Bowen and Vilner, 1994; Vilner et al., 1995a). In an attempt to determine the mechanisms that might be involved in the cytotoxic effects of sigma ligands and potential signaling pathways used by sigma-receptors in normal cell functions, we investigated whether sigma ligands can modulate the levels of intracellular Ca^{2+} . Previous experiments revealed that sigma ligands from various structural classes produce a dual modulation of $[\text{Ca}^{2+}]_i$: release of Ca^{2+} from intracellular stores and blockade of depolarization-dependent influx of Ca^{2+} (Vilner and Bowen, 1995). Here, we further characterize the sigma ligand-induced intracellular release of Ca^{2+} in human SK-N-SH neuroblastoma cells with respect to specificity, time course, Ca^{2+} source, and pharmacological profile and show that it is mediated via sigma-2 receptors. Portions of this work have been published previously in abstract form (Vilner and Bowen, 1995; Bowen et al., 1996).

Materials and Methods

Cell Culture. SK-N-SH line cell (human neuroblastoma, HTB-11; American Type Culture Collection, Manassas, VA) were grown in 75-cm² culture flasks (Costar, Cambridge, MA) in Dulbecco's modified Eagle's medium (DMEM), enriched with 10% FBS in a humidified atmosphere of 5% CO_2 /95% air at 37°C. Once cells formed a confluent monolayer, they were washed in Dulbecco's phosphate-buffered saline [DPBS; 136.9 mM NaCl, 2.68 mM KCl, 0.49 mM MgCl_2 , 8.10 mM Na_2HPO_4 , 1.47 mM KH_2PO_4 , 0.904 mM CaCl_2 , 5.55 mM D-(+)-glucose, 0.33 mM sodium pyruvate, 0.05 g/l streptomycin

sulfate, pH 7.2] and dispersed with cell dissociation solution (2 ml/flask for 10 min at 37°C). Cells were then harvested by centrifuging and resuspending in DMEM/10% FBS at a density of 50,000 to 100,000 cells/ml.

Cells in 1.5 ml of medium were plated onto round, 25-mm-diameter cover glasses that had been previously covered with dry collagen (Vitrogen100; Celtrix Pharmaceuticals, Inc., Santa Clara, CA) as follows. The cover glasses were placed in round, 35-mm-diameter tissue culture dishes. Sterile collagen (1 ml) was diluted with 32 ml of sterile 33% ethanol, and two or three drops of this solution were dispersed over the cover glass and allowed to dry for ≥ 1 h before plating cells. After 2 to 3 days in culture, cells were used for Ca^{2+} measurement as described later.

As is well established, SK-N-SH cultures consisted of two different cell types: neuron-like cells and epithelium-like cells (Ross et al., 1983). The two cell types are easily distinguishable using phase-contrast microscopy on the basis of shape and size. Neuron-like cells are small (15–25 μm) and have a polygonal shape; a dark cytoplasm with a large, bright, round nucleus (often two per cell); and one to three dark nucleoli. After 2 to 3 days in culture, neuron-like cells form easily visible processes. In general, the neuron-like cells lay on a background formed by the epithelium-like cells. Epithelium-like cells are much larger (three to five times the size of neuron-like cells) and have a spindle-shaped cytoplasm that is much lighter than that in neuron-like cells. These cells contain a small nucleus with one or two nucleoli. Epithelium-like cells often form pseudopodia, which are easily distinguished from the processes formed by neuron-like cells. The proportion of neuron-like cells to epithelium-like cells was different in different passages and was found to be 50:50, 60:40, or 70:30. For the experiments described here, only neuron-like cells were chosen and used for Ca^{2+} measurements.

Alternatively, SK-N-SH cell cultures were developed that were $>90\%$ enriched in the neuron-like cell type. To achieve enriched cultures, mixed cells were cultured in flasks to ~ 60 to 70% confluence. After decanting of the medium, the culture was washed with sterile DPBS two or three times. Nonenzymatic cell dissociation solution (Sigma Chemical Co., St. Louis, MO) was added to culture for 3 to 5 min. Because neuron-like cells lie on the surface of epithelium-like cells, they detach more easily and earlier. The detached cells were collected and centrifuged (2000 rpm, 5–7 min), and the cell pellet was resuspended and replated in fresh medium. The cells were again allowed to grow to 60 to 70% confluence, and the procedure was repeated. Cells were frozen as stocks in 90% medium/10% DMSO. After reculture, cells were used for experiments. Cultures prepared in this way consisted of 90 to 95% neuron-like cells.

$[\text{Ca}^{2+}]_i$ Measurement in Single Cells. For $[\text{Ca}^{2+}]_i$ measurement, cover glasses containing cells were washed twice in DPBS and then incubated in DPBS containing 2.0 to 3.3 μM Indo-1 AM and 0.066% Pluronic F-127 (Molecular Probes, Inc., Eugene, OR). After incubation for 60 to 75 min at room temperature in darkness, cultures were washed twice in DPBS and allowed to stand at room temperature in the dark for ≥ 30 min until use in the experiments. Cells could be maintained this way for up to 5 h before use, with no differences in basal Ca^{2+} levels or results.

Cover glasses containing cells were mounted in Leuden coverslip dishes (Medical Systems Co., Greenvale, NY) with 0.25 ml of DPBS, and the dish then mounted on the stage of a Nikon DIAPHOT-TMD inverted microscope equipped with a dual emission P1 photometer (Nikon, Melville, NY). Cells were examined with a 40 $\times$ oil immersion objective (Fluor 40/1.30 oil PH4DU; Nikon) in phase contrast or fluorescence manner.

For Indo-1 excitation, the light beam was reduced using a neutral density filter (by 1/16 or 1/32). After passing through a 340-nm interference filter, the excitation light beam reflected from a 400 DM dichroic mirror through the diaphragm opening, the size of which was chosen to be smaller or equal to the size of the cell under examination. The emission light beam from cells was split by a 455 DM dichroic mirror to 410- and 485-nm interference filters. Each

beam was monitored by an individual photomultiplier, and the intensity ratio (410:485 nm) was calculated on-line using an SACON (IBM compatible) computer at 360-ms intervals with the FASTINCA Ca^{2+} measurement program (University of Cincinnati Medical Center, Cincinnati, OH).

A standard curve was used to derive experimental $[\text{Ca}^{2+}]_i$ values. The standard curve was generated by using various concentrations of Ca^{2+} (Calcium Calibration Buffer Kit) in the presence of indicator dye Indo-1 (Molecular Probes). During each experiment, background fluorescence was estimated on a chamber area without cells, and this value was automatically subtracted from the measured emission of each channel. The ratios of cell emission were compared with the standard curve stored in the computer program, and both the ratios and $[\text{Ca}^{2+}]_i$ were displayed on screen.

Before each experiment, the particular neuron-like cell to be examined on a given dish was chosen from the overall field of cells in phase contrast mode. Preliminary measurements of $[\text{Ca}^{2+}]_i$ were taken on various cells present in the field (before any drug additions). The basal $[\text{Ca}^{2+}]_i$ in normal cells is in the range of 80 to 120 nM (Clapham, 1995). Only cells with basal $[\text{Ca}^{2+}]_i$ in this range were chosen for the experiments described here.

All measurements were performed in DPBS or, where specified, in Ca^{2+} -free DPBS. Unless specified otherwise, DPBS was at pH 7.2. Where specified, some experiments were carried out in DPBS at pH 8.2 (DPBS was adjusted to pH 8.2 with 1 M NaOH). Drugs were added to cells in the following manner. To the Leuden dish containing the coverglass-laden cells in 0.25 ml of DPBS was added 0.25 ml of DPBS containing the compound or compounds at $2\times$ the desired final concentration. Experimental measurements were taken in this final volume of 0.5 ml of DPBS. For short time course experiments, only one cell was examined per dish in an experimental condition. For prolonged time course experiments, readings were sometimes taken from several individual cells in the same field. For each experimental condition, measurements from several cells from different batches were averaged and expressed with S.E. values.

Test compounds were stored at -25°C at a stock concentration of 10 mM, made up in 10 or 20 mM HCl or in 100% DMSO. Thapsigargin was stored as a 5 mM stock in 100% DMSO. Carbachol was made up as a 1 M stock in water.

Sigma Receptor Binding. Sigma receptor binding was carried out in membranes from SK-N-SH neuroblastoma cells. For binding assays, cells were plated at a density of 2.5×10^6 cells/75-cm² plastic culture flasks and cultured under the conditions described above. The medium was changed once a week, and the cells were allowed to grow to near confluence. Cells were harvested and membranes were prepared as described previously (Vilner et al., 1995b), with the following modifications. Cells were washed in situ with DPBS before detachment and then detached by scraping in DPBS. The final membrane pellet was resuspended to a protein concentration of 10 to 15 mg/ml in 10 mM Tris · HCl, pH 7.4.

Sigma-1 receptors were labeled using the sigma-1-selective probe (+)-[³H] pentazocine (Bowen et al., 1993a). SK-N-SH neuroblastoma cell membranes (100–150 μg of membrane protein) were incubated with 10 nM (+)-[³H]pentazocine in a total volume of 0.25 ml of 50 mM Tris · HCl, pH 8.3. Incubations were carried out for 120 min at 37°C . Nonspecific binding was determined in the presence of 10 μM unlabeled haloperidol. Assays were terminated by dilution with 5 ml of ice-cold 10 mM Tris · HCl, pH 8.0, and vacuum filtration through glass fiber filters using a Brandel cell harvester (Gaithersburg, MD). Filters were then washed twice with 5 ml of ice-cold 10 mM Tris · HCl, pH 8.0. Filters were counted in CytoScint cocktail (ICN, Costa Mesa, CA) after an overnight extraction of counts. Filters were soaked in 0.5% polyethyleneimine for ≥ 30 min at 25°C before use.

Sigma-2 receptors were labeled using [³H]DTG, under conditions in which sigma-1 receptors are masked with dextrallorphan (Hellewell et al., 1994). SK-N-SH neuroblastoma cell membranes (100–150 μg of membrane protein) were incubated with 10 nM [³H]DTG in the presence of 1 μM dextrallorphan. Nonspecific bind-

ing was determined in the presence of 10 μM unlabeled haloperidol. Incubation conditions and other manipulations were as described above for the sigma-1 receptor assay.

Chemicals. (+)-[³H]Pentazocine (51.7 Ci/mmol) was synthesized as described previously (Bowen et al., 1993a). [³H]DTG (35.2 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA).

Haloperidol, serotonin, atropine, ibogaine, Tris · HCl, polyethyleneimine, DMEM, DPBS, and cell dissociation solution were purchased from Sigma Chemical Co. FBS was purchased from Atlanta Biologicals (Norcross, GA). DTG was purchased from Aldrich Chemical Co. (Milwaukee, WI). (+)-Pentazocine, (–)-naltrexone, and morphine were kindly provided by Dr. Kenner C. Rice (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD). Dextrallorphan and (+)-SKF-10,047 [(+)-N-allylnormetazocine] were provided by Dr. F. I. Carroll (Research Triangle Institute, Research Triangle Park, NC). Thapsigargin was purchased from Research Biochemicals Inc. (Natick, MA).

Novel sigma ligands based on the aryl ethylenediamine pharmacophore were synthesized as described previously (Bowen et al., 1992; de Costa et al., 1992a,b) as hydrochloride or hydrobromide salts and have code names and chemical names as follows: BD737, (1*S*,2*R*)-*cis*-N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(1-pyrrolidinyl)-cyclohexylamine; JL-II-147, 2-[N-[2-(1-pyrrolidinyl)ethyl]-N-methylamino]-6,7-dichlorotetralin; BD1008, N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(1-pyrrolidinyl)ethylamine; LR172, N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(1-homopiperidinyl)ethylamine; BD1047, N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino)ethylamine; BD1063, 1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine; and BD1006, N-methyl-2-(1-pyrrolidinyl)ethylamine. 5-Phenylmorphane-related compounds were synthesized as previously described (Bertha et al., 1995) as perchlorate salts and have the following code names: CB-64D, (+)-(1*R*,5*R*)-*E*-8-benzylidene-5-(3-hydroxyphenyl)-2-methylmorphane-7-one; CB-64L, (–)-(1*S*,5*S*)-*E*-8-benzylidene-5-(3-hydroxyphenyl)-2-methylmorphane-7-one; CB-53D, (–)-(1*R*,5*S*)-5-(3-hydroxyphenyl)-2-methylmorphane-7-one. The following were purchased from Research Biochemicals Inc.: 4-(4-chlorophenyl)- α -4-fluorophenyl-4-hydroxy-1-piperidinebutanol [reduced haloperidol (Red HAL)], (–)-sulpiride, mianserin, ritanserin, [D-Ala², N-Me-Phe⁴, Gly-ol⁵]-enkephalin (DAMGO), and (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine [(+)-MK-801 hydrogen maleate]. Noribogaine was synthesized through the demethylation of ibogaine (Dr. Craig Bertha, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD).

Results

Acute Effect of Sigma Ligands on Intracellular Ca^{2+} Levels

Effect of Selective Sigma Ligands. Human SK-N-SH neuroblastoma cells, which have been shown to express both sigma-1 and sigma-2 receptors (Vilner et al., 1995b), were used to examine the direct effect of sigma ligands on intracellular Ca^{2+} . Figure 1 shows time versus $[\text{Ca}^{2+}]_i$ traces for the effect of the selective sigma agonist BD737 (Bowen et al., 1992) and the benzylidene-5-phenylmorphane CB-64D (Bowen et al., 1995a) at 100 μM . Both BD737 and CB-64D produced a transient rise in $[\text{Ca}^{2+}]_i$ that began in seconds after the addition of drug to cells. The peak level of $[\text{Ca}^{2+}]_i$ was usually reached in 1.5 to 3 min and returned to a baseline level that was slightly higher than the original within 4 to 5 min after reaching the peak. Red HAL, a sigma-active metabolite of haloperidol, produced the same effect (plot not shown).

The dose dependence of this effect was investigated, and the results are shown in Fig. 2. BD737, Red HAL, and CB-

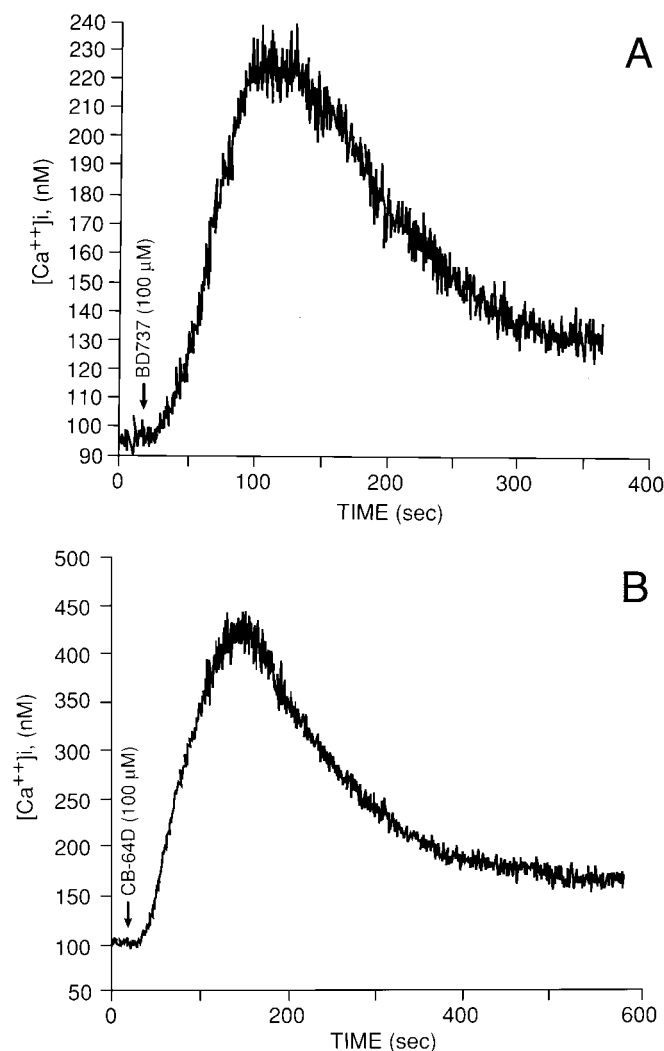


Fig. 1. Acute effect of sigma ligands on $[Ca^{2+}]_i$ in human SK-N-SH neuroblastoma cells. Sigma ligands ($100 \mu M$) were added to cells in normal DPBS at the indicated time point, and the effect on $[Ca^{2+}]_i$ was monitored until a return to stable baseline. Ratios of 410:485 nm were converted to concentration (nM) using the FASTINCA program as described in *Materials and Methods*. Representative time-versus- $[Ca^{2+}]_i$ traces for BD737 (A) and CB-64D (B) are shown. The sigma ligands produced a transient rise in $[Ca^{2+}]_i$ that began within seconds after drug addition and returned to baseline within 7 to 10 min.

64D produced dose-dependent increases in $[Ca^{2+}]_i$, with CB-64D producing the greatest response. Significant increases in $[Ca^{2+}]_i$ were observed for BD737 and CB-64D at concentrations as low as $3 \mu M$.

Sigma-1 and Sigma-2 Receptors of SK-N-SH Neuroblastoma Cells and Pharmacological Profile of Effect on $[Ca^{2+}]_i$. As previously reported (Vilner et al., 1995b), the sigma-1 receptor probe (+)- $[^3H]$ pentazocine labeled a single population of sites with a K_d value of 28.0 nM and a B_{max} value of 975 fmol/mg protein in membranes from SK-N-SH neuroblastoma cells. The sigma-2 receptor probe $[^3H]$ DTG (in the presence of dextrallorphan to mask sigma-1 sites) labeled a single population of sites with a K_d value of 32.4 nM and a B_{max} value of 944 fmol/mg protein.

The binding affinities of various ligands at sigma-1 and sigma-2 receptors in membranes from human SK-N-SH neuroblastoma cells are shown in Table 1. The competition pro-

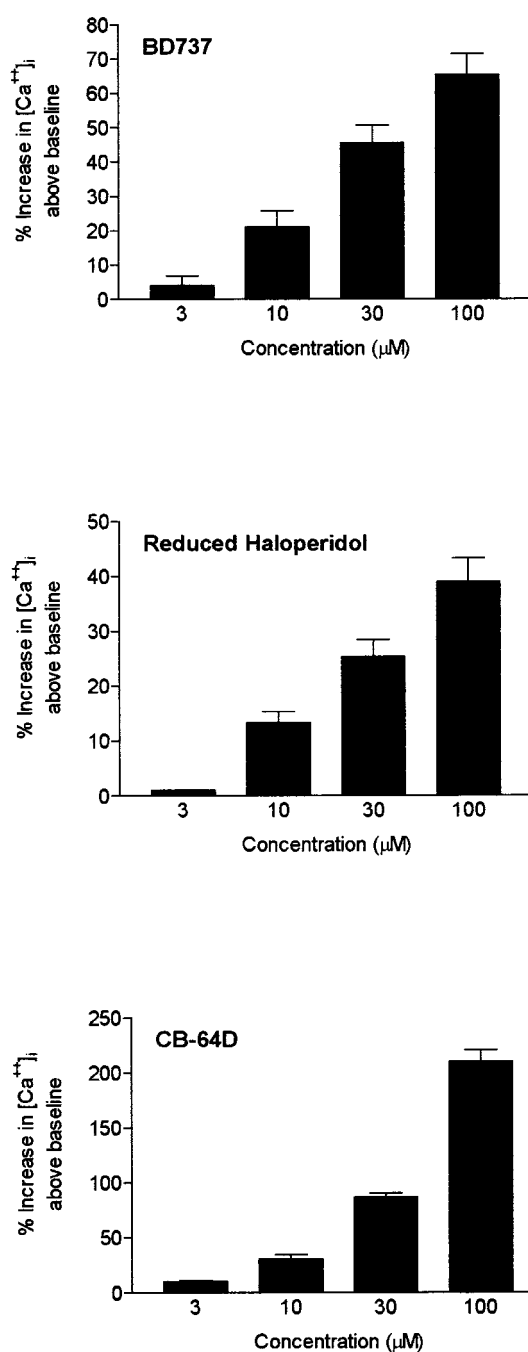


Fig. 2. Dose-response for ability of sigma ligands to increase $[Ca^{2+}]_i$. Various concentrations (3, 10, 30, and $100 \mu M$) of BD737, Red HAL, and CB-64D were added to cells in normal DPBS, and time-versus- $[Ca^{2+}]_i$ traces were obtained as shown in Fig. 1. The percent increase in $[Ca^{2+}]_i$ produced by the ligand was calculated for each individual trace by determining the peak (maximum) Ca^{2+} concentration relative to the starting baseline level. One cell per dish was chosen for study, and each cell was used only once. The initial (basal) $[Ca^{2+}]_i$ was 80 to 120 nM. Cells with higher levels of basal $[Ca^{2+}]_i$ were not used in this study. Values are the averages of no fewer than three cells $\pm$ S.E.

file of ligands at sigma-1 receptors labeled with (+)- $[^3H]$ pentazocine in SK-N-SH membranes was similar to that observed in our standard assay in guinea pig brain membranes (Bowen et al., 1993a). Likewise, the profile obtained at sigma-2 receptors labeled with $[^3H]$ DTG (in the presence of dextrallorphan) was generally similar to that obtained in

TABLE 1

Sigma Receptor Binding in SK-N-SH Neuroblastoma Cells and Pharmacological Profile of Effect of Sigma Ligands on $[Ca^{2+}]_i$

Sigma receptor binding assays were carried out under the conditions described in *Materials and Methods*. Twelve concentrations of unlabeled test ligand ranging from 0.05 to 10,000 nM or 0.5 to 100,000 nM were incubated with SK-N-SH neuroblastoma cell membranes and 10 nM [3H](+)-pentazocine (sigma-1 receptors) or 10 nM [3H]DTG in the presence of 1 μ M dextrallorphan (sigma-2 receptors). IC_{50} values were determined using the iterative curve-fitting program GraphPAD Prism (San Diego, CA). IC_{50} values were then converted to apparent K_i values using the Cheng-Prusoff equation and radioligand K_d values determined previously (Vilner et al., 1995b). K_i values are the averages of two to three experiments $\pm$ S.E.M. Each experiment was carried out in duplicate. Values for some compounds have been published previously (Vilner et al., 1995b). The various compounds were examined for their ability to affect $[Ca^{2+}]_i$ at a concentration of 100 μ M. Experiments were carried out and data analyzed as described in the legend to Fig. 2. All data are the averages from three cells $\pm$ S.E.M. The following ligands for other receptors, which lack affinity for sigma-1 or sigma-2 receptors produced little or no change ($<10\%$) in $[Ca^{2+}]_i$ ($n = 2-3$ cells): morphine (30 and 100 μ M), DAMGO (1 μ M), (-)-naltrexone (100 μ M), atropine (100 μ M), (-)-sulpiride (30 and 100 μ M), MK-801 (30 and 100 μ M), and mianserin (10 μ M).

Compound	K_i		% Increase in $[Ca^{2+}]_i$ above Basal at 100 μ M
	Sigma-1	Sigma-2	
	nM		
CB-64D	5,304 $\pm$ 313	60.8 $\pm$ 7.49	210 $\pm$ 11
CB-64L	102 $\pm$ 17	759 $\pm$ 22	28.5 $\pm$ 1.1
JL-II-147	41.4 $\pm$ 1.3	120 $\pm$ 2	108 $\pm$ 8
BD737	8.78 $\pm$ 1.54	68.3 $\pm$ 14.0	65.3 $\pm$ 6.14
LR172	6.10 $\pm$ 0.20	13.9 $\pm$ 0.2	63.0 $\pm$ 4.97
BD1008	1.24 $\pm$ 0.21	32.5 $\pm$ 1.50	42.7 $\pm$ 3.30
Haloperidol	6.44 $\pm$ 0.15	221 $\pm$ 29	52.0 $\pm$ 9.63
Red HAL	47.2 $\pm$ 16.3	123 $\pm$ 20	39.0 $\pm$ 4.25
Ibogaine	31,696 $\pm$ 7,651	963 $\pm$ 68	40.5 $\pm$ 2.0
DTG	203 $\pm$ 7	58.4 $\pm$ 8.5	8.0 $\pm$ 0
(+)-Pentazocine	16.7 $\pm$ 0.95	6,611 $\pm$ 1,042	8.0 $\pm$ 0.57
(-)-Pentazocine	807 $\pm$ 99	2,324 $\pm$ 225	0 $\pm$ 0
(+)-SKF 10,047	597 $\pm$ 139	39,740 $\pm$ 4,328	6.0 $\pm$ 2.5
(-)-SKF 10,047	50,399 $\pm$ 801	41,461 $\pm$ 5,801	5.5 $\pm$ 3.9
Dextrallorphan	125 $\pm$ 12	8,393 $\pm$ 1,323	9.0 $\pm$ 0
CB-53D	>100,000	>77,000	8.7 $\pm$ 0.54
BD1006	21,628 $\pm$ 1,452	>86,000	0 $\pm$ 0
Noribogaine	6,293 $\pm$ 287	6,166 $\pm$ 10	0 $\pm$ 0

our standard assay in rat liver membranes (Hellewell et al., 1994). The aryethylene diamines BD1008, BD737, LR172, and JL-II-147 (de Costa et al., 1992a,b; Bowen et al., 1992), bound with high affinity to both sigma-1 and sigma-2 receptors of SK-N-SH neuroblastoma cells, as was observed with sigma-1 receptors of guinea pig brain and sigma-2 receptors of rat liver.

However, as reported previously when rodent-derived tumor cell lines were compared with human cell lines from various tissues (Vilner et al., 1995b), there are some affinity differences that might suggest heterogeneity or species differences in sigma-2 sites when SK-N-SH neuroblastoma sigma-2 receptors are compared with those of rat liver membranes (Hellewell et al., 1994). At the SK-N-SH sigma-2 site, haloperidol and (-)-pentazocine have substantially lower affinity compared with sigma-2 sites of rat liver. Although (+)-benzomorphans and (+)-morphinans [(+)-pentazocine, (+)-SKF-10,047, and dextrallorphan] have very low affinity for the SK-N-SH sigma-2 site compared with the sigma-1 sites in these cells, there is decreased enantioselectivity for (-)-isomers over (+)-isomers at the sigma-2 sites. For example, although still 3-fold enantioselective, the decreased affinity for (-)-pentazocine eliminated much of the sigma-2 enantioselectivity for (-)-pentazocine over (+)-pentazocine seen in rat liver membranes and membranes from rodent cell lines (Hellewell and Bowen, 1990; Hellewell et al., 1994; Vilner et al., 1995b).

Despite these differences, however, it is clear that the pharmacological characteristics that distinguish sigma-2 receptors from sigma-1 receptors in other tissues are maintained in human SK-N-SH neuroblastoma cells. (+)-Pentazocine, (+)-SKF-10,047, and dextrallorphan exhibit 396-, 66.5-, and 67.1-fold selectivity, respectively, for sigma-1 sites over sigma-2 sites in SK-N-SH neuroblastoma cells. However, (-)-pentazocine fails to strongly distinguish sigma-1 and sigma-2 sites (2.9-fold preference for sigma-2 sites), and (-)-SKF-10,047 has negligible affinity at both sites. Strong preference of sigma-1 receptors for (+)-benzomorphans compared with sigma-2 receptors and the relative inability of most (-)-benzomorphans to distinguish the two sites are hallmarks of sigma subtypes (Hellewell and Bowen, 1990; Quirion et al., 1992). The 5-phenylmorphans CB-64D and the iboga alkaloid ibogaine have previously been shown to exhibit marked selectivity for sigma-2 receptors over sigma-1 sites (Bowen et al., 1995a,b). Although CB-64D and ibogaine showed 3.7- and 4.8-fold lower affinity at sigma-2 sites of SK-N-SH cells compared with rat liver membranes, these compounds maintain 87- and 33-fold selectivity for sigma-2 sites of SK-N-SH cells over sigma-1 sites of these cells. Furthermore, as demonstrated in the initial study using the standard assay of membranes from rat liver and guinea pig brain (Bowen et al., 1995a), sigma-2 sites of SK-N-SH neuroblastoma cells exhibit enantioselectivity for CB-64D [the (+)-isomer] over CB-64L [the (-)-isomer], whereas sigma-1 sites exhibit opposite enantioselectivity.

Along with binding affinities of various compounds at sigma-1 and sigma-2 receptors, Table 1 shows the effect on $[Ca^{2+}]_i$ produced by a 100 μ M concentration of each compound. The ability of compounds to produce a rise in $[Ca^{2+}]_i$ correlated with their binding activity at sigma-2 receptors but not sigma-1 sites. In addition to the compounds shown in Figs. 1 and 2, the butyrophenone haloperidol; the aryl ethylene diamine-related compounds LR172, JL-II-147, and BD1008; and the iboga alkaloid ibogaine were also effective at increasing $[Ca^{2+}]_i$. However, the (+)-benzomorphans (+)-pentazocine, (+)-SKF-10,047, and dextrallorphan produced only very small effects relative to the other sigma ligands. Surprisingly, the aryl guanidine DTG also had very little effect on $[Ca^{2+}]_i$.

Of the sigma ligands tested, (+)-pentazocine, (+)-SKF 10,047, dextrallorphan, CB-64D, and ibogaine are the only compounds that show marked selectivity for one or the other sigma subtype (Table 1), with the other active sigma ligands having high to moderate affinity for both sigma receptor subtypes. Although the K_i values determined under the non-physiological conditions of the sigma ligand binding assay are in the nanomolar range and sigma ligands showed effects on $[Ca^{2+}]_i$ in the micromolar range, the high potency of CB-64D relative to the very low potency of (+)-pentazocine and the other (+)-benzomorphans strongly suggests that this effect is mediated by sigma-2 receptors. This is also supported by the activity of ibogaine, which is also selective for sigma-2 sites over sigma-1. In addition, support for mediation by sigma-2 receptors comes from the activity of enantiomeric pairs. As described above, both (-)-pentazocine and (-)-SKF-10,047 have affinities at SK-N-SH neuroblastoma sigma-2 receptors too low to be expected to produce effects on $[Ca^{2+}]_i$, and they do not. However, a comparison of the 5-phenylmorphans reveals that 100 μ M CB-64D is 7.4-fold

more effective than CB-64L, which correlates favorably with the 12.5-fold higher binding affinity of CB-64D at sigma-2 receptors compared with CB-64L. Because CB-64D and CB-64L show the reverse enantioselectivity at sigma-1 sites, mediation by sigma-2 receptors is strongly supported.

Specificity of Effect on $[Ca^{2+}]_i$ for Sigma Receptors.

The specificity of the effect for sigma-2 receptors was further investigated. BD1006 is an ethylene diamine that lacks an aromatic ring moiety. As shown in Table 1, this compound is devoid of sigma receptor affinity and thus is a control for possible nonspecific effects of diamines. CB-53D is a close analog of the 5-phenylmorphans CB-64D and CB-64L but lacks the benzylidene moiety that imparts sigma binding affinity (Bertha et al., 1995). Noribogaine is the *O*-desmethyl derivative of ibogaine and has markedly reduced sigma receptor affinity relative to ibogaine (Bowen et al., 1995b). All three of these compounds produced <10% increase in $[Ca^{2+}]_i$ at a concentration of 100 μ M (Table 1). Thus, structurally related compounds that lack significant sigma receptor affinity failed to produce robust effects. This supports the notion that the activity of the arylethylenediamines BD737, LR172, BD1008, and JL-II-147; of the benzylidene phenylmorphans CB-64D and CB-64L; and of the iboga alkaloid ibogaine is related to the sigma-2 receptor binding affinity rather than to a nonspecific effect.

The ligands used in the current study are relatively selective for sigma receptors over other types of neurotransmitter receptors, with the exception of CB-64D, which also has high affinity for μ -opioid receptors (Bertha et al., 1995; Bowen et al., 1995a). However, to investigate whether the effect of sigma ligands might be mediated by high-dose interaction with other neurotransmitter receptors that might be present on SK-N-SH cells, agonists or antagonists for other receptors known to cross-react with some sigma ligands were examined. Morphine, (-)-naltrexone, atropine, (-)-sulpiride, (+)-MK-801, and mianserin all lack significant affinity for sigma-1 and sigma-2 sites ($K_i > 10,000$ nM). Morphine (30 and 100 μ M), DAMGO (1 μ M), (-)-naltrexone (100 μ M), atropine (100 μ M), (-)-sulpiride (30 and 100 μ M), (+)-MK-801 (30 and 100 μ M), and mianserin (10 μ M), all produced either no response or <10% rise in $[Ca^{2+}]_i$ within 10 min after the addition of the compound ($n = 2$ or 3 cells). This shows that sigma ligands are not acting by blocking opiate, muscarinic cholinergic, dopamine D_2 , NMDA glutamatergic, or serotonin receptors. Furthermore, the lack of effect of morphine and DAMGO shows that CB-64D is not acting by activation of opiate receptors, despite its high affinity for μ -opioid receptors.

Serotonin (1 mM) produced a very rapid and transient $313 \pm 41\%$ increase ($n = 4$ cells) in $[Ca^{2+}]_i$. This is presumably the result of the activation of serotonin₂ receptors present on these cells and the stimulation of phosphoinositide turnover because this effect of serotonin was not diminished by the removal of extracellular Ca^{2+} , as it would have been if serotonin₃ receptors (a ligand-gated cation channel) were involved. To determine whether sigma ligands might be acting as serotonin receptor agonists, the effect of serotonin antagonists on the action of CB-64D was examined. The serotonin antagonists mianserin and ritanserin had no effect on the rise in $[Ca^{2+}]_i$ produced by CB-64D (values are percent rise in $[Ca^{2+}]_i$ above baseline): 30 μ M CB-64D alone, $47.5 \pm 5.3\%$ ($n = 2$); 10 μ M mianserin + 30 μ M CB-64D, $51.0 \pm 7.9\%$

($n = 3$); and 10 μ M ritanserin + 30 μ M CB-64D, $50.0 \pm 3.8\%$ ($n = 3$). These results show that sigma ligands are not producing their effect by acting as agonists at serotonin receptors. Thus, although micromolar concentrations of sigma ligands are required to produce the described effect on $[Ca^{2+}]_i$, the effect is not due to actions at other neurotransmitter receptors known to bind sigma ligands or to nonspecific effects of the compounds.

Blockade of Ligand-Induced Rise in $[Ca^{2+}]_i$ by Sigma Receptor Antagonists BD1047 and BD1063. The arylethylenediamines BD1047 and BD1063 have been demonstrated as highly selective sigma receptor antagonists (Matsumoto et al., 1995). BD1047 binds to sigma-1 and sigma-2 receptors of human SK-N-SH neuroblastoma cells with a K_i value of 36.5 ± 2.2 and 587 ± 29 nM, respectively. BD1063 binds to SK-N-SH sigma-1 and sigma-2 receptors with a K_i value of 8.80 ± 0.1 and 332 ± 40 nM, respectively. The effects of BD1047 and BD1063 on $[Ca^{2+}]_i$ were investigated.

We have shown that the potency of sigma-2 ligands at producing the transient rise in $[Ca^{2+}]_i$ is markedly increased when the extracellular pH is raised from 7.2 to 8.2, suggesting that deprotonation (and increased lipid solubility) enhances the activity of the compounds (Bowen et al., 1999). Under these conditions, BD1047 and BD1063 can be shown to efficiently attenuate the sigma ligand-induced transient

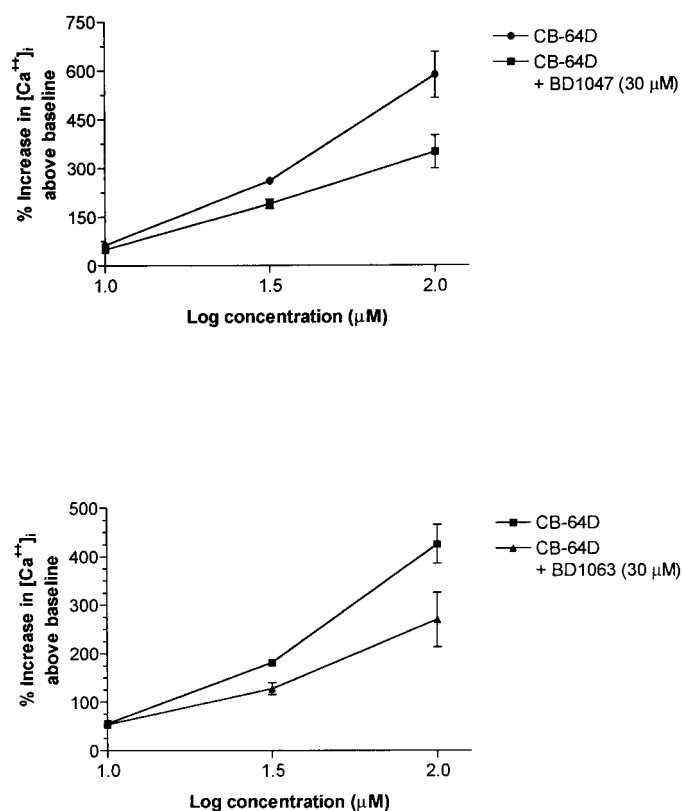


Fig. 3. Effect of the sigma antagonists BD1047 and BD1063 on ability of CB-64D to increase $[Ca^{2+}]_i$. BD1047 (30 μ M) or BD1063 (30 μ M) was added to SK-N-SH neuroblastoma cells simultaneously with 10, 30, or 100 μ M CB-64D. Effect on $[Ca^{2+}]_i$ was then monitored for 10 min, and percent increase in $[Ca^{2+}]_i$ was calculated as described in the legend to Fig. 2. Values are the averages of no fewer than three cells $\pm$ S.E. These experiments were carried out in DPBS at pH 8.2. Under these conditions, the rise in $[Ca^{2+}]_i$ produced by 30 μ M BD1047 and BD1063 alone was 31.4 ± 5.06 and $25 \pm 2.18\%$, respectively.

rise in $[Ca^{2+}]_i$. The results are shown in Figs. 3 and 4. At a concentration of 30 μ M, BD1047 and BD1063 alone produced a rise in $[Ca^{2+}]_i$ of only 31.4 ± 5.06 and $25 \pm 2.18\%$, respectively (compare with 18.0 ± 1.90 and $2.66 \pm 2.17\%$, respectively, at pH 7.2). The low activity of these compounds, despite moderate sigma-2 affinity, is consistent with antagonist or partial agonist activity.

Figure 3 shows dose-response curves for CB-64D carried out in the absence and presence of 30 μ M BD1063 (top) or 30 μ M BD1047 (bottom), with antagonists added to cells simultaneously with the putative agonist. The activity of CB-64D at all concentrations was attenuated with both antagonists. There initially appeared to be little attenuation by either antagonist at the 10 μ M concentration of CB-64D. However, the stimulation produced by 30 μ M antagonist alone ($\sim 30\%$) and the stimulation produced by 10 μ M CB-64D alone ($\sim 60\%$) was less than additive when antagonist and CB-64D were combined (stimulation, $\sim 50\%$), indicating antagonism.

More dramatic blockade by the two sigma antagonists was observed when the cells were preincubated with the antagonist, followed by the addition of the putative agonist. Figure 4 (top) shows the effect of 100 μ M CB-64D before and after cells were preincubated for 10 min with 30 μ M BD1047. Figure 4 (bottom) shows the same for 100 μ M BD737 and 30 μ M BD1063. Under these conditions, BD1047 inhibited the activity of CB-64D by 79%. This compares with 40% inhibition for 30 μ M BD1047 versus 100 μ M CB-64D without

preincubation (Fig. 3). Likewise, BD1063 inhibited the activity of BD737 by 69%.

The ability of BD1047 and BD1063 to block the activity of CB-64D and BD737 confirms that active compounds are acting as sigma-2 receptor agonists. The demonstration of this agonist-antagonist relationship for producing a rise in $[Ca^{2+}]_i$ supports the notion that sigma-2 binding sites in SK-N-SH neuroblastoma cells are functioning as classical receptors.

Origin of Transient Increase in $[Ca^{2+}]_i$ Induced by Sigma Ligands. To determine whether extracellular Ca^{2+} contributed to the transient rise in $[Ca^{2+}]_i$, the effect of removing extracellular Ca^{2+} was investigated as shown in Table 2. Depolarization of SK-N-SH cells with 55 mM KCl in normal DPBS (which contains ~ 1 mM $CaCl_2$) produced a robust increase (565%) in $[Ca^{2+}]_i$ due to a large influx of Ca^{2+} through voltage-gated Ca^{2+} channels. Placement of cells in Ca^{2+} -free DPBS completely eliminated this influx, confirming the absence of extracellular Ca^{2+} under these conditions. However, removal of extracellular Ca^{2+} had little or no effect on the ability of 100 μ M BD737, Red HAL, or CB-64D to increase $[Ca^{2+}]_i$. This shows that sigma ligands produce a rise in $[Ca^{2+}]_i$ by release from intracellular stores.

To investigate the subcellular origin of the Ca^{2+} comprising the immediate, transient rise in $[Ca^{2+}]_i$, SK-N-SH neuroblastoma cells were treated with thapsigargin. Thapsigargin depletes the intracellular store of Ca^{2+} from the endoplasmic reticulum by irreversibly inhibiting the sarcoplasmic-endoplasmic reticulum Ca^{2+} -ATPase (SERCA), which is responsible for sequestration of Ca^{2+} in the lumen (Clapham, 1995). Carbachol is known to stimulate muscarinic receptors in SK-N-SH neuroblastoma cells, causing hydrolysis of inositol lipids, formation of IP_3 , and subsequent release of Ca^{2+} from the endoplasmic reticulum via activation of the IP_3 receptor (Fisher and Snider, 1987). This effect is sensitive to thapsigargin and thus was used as a control to confirm the effectiveness of the thapsigargin treatment in the current study. Table 3 shows that 1 mM carbachol produced a 20-fold increase in $[Ca^{2+}]_i$ in these

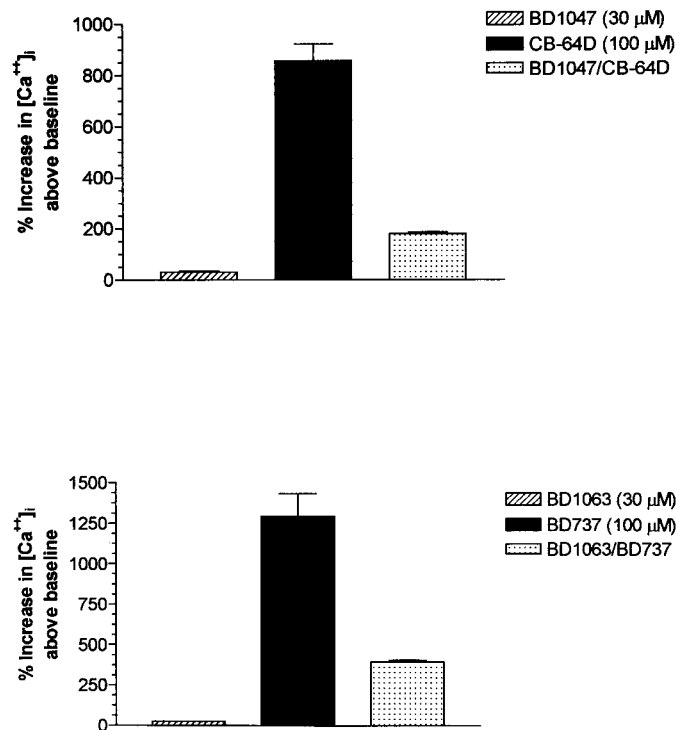


Fig. 4. Effect of sigma antagonists on action of CB-64D and BD737 after preincubation of cells with antagonists. Cells were treated with 30 μ M BD1047 for 10 min before the addition of 100 μ M CB-64D (top) or with 30 μ M BD1063 for 10 min before the addition of 100 μ M BD737 (bottom) in DPBS at pH 8.2. Effect on $[Ca^{2+}]_i$ was then monitored for 10 min after the addition of the agonist. Antagonists remained present during incubation with agonists. Values were compared with cells treated with either antagonist alone (30 μ M) or agonist alone (100 μ M), with $[Ca^{2+}]_i$ monitored for 10 min after the addition in each case. Percent increase in $[Ca^{2+}]_i$ was calculated as described in the legend to Fig. 2. Values are the averages from two to five cells $\pm$ S.E.

TABLE 2

Effect of Extracellular Calcium Removal on Acute Response to Sigma Ligands

Experiments were carried out as described in the legend to Fig. 1 using either normal DPBS (which contains 0.904 mM $CaCl_2$) or Ca^{2+} -free DPBS. Values are expressed as percent change in $[Ca^{2+}]_i$ relative to basal level, calculated as described in the legend to Fig. 2. Data with 55 mM KCl is included as control for effective removal of extracellular Ca^{2+} and examines the ability of K^+ to stimulate entry of Ca^{2+} through voltage-dependent Ca^{2+} channels known to be present in these cells. KCl value for normal DPBS is the average of 56 cells $\pm$ S.E.M. and value for Ca^{2+} -free DPBS is the average of 22 cells $\pm$ S.E.M. Values for the sigma ligands BD737, reduced haloperidol, and CB-64D were determined at a concentration of 100 μ M and are the averages of three cells $\pm$ S.E.M.

Condition	% Increase in $[Ca^{2+}]_i$ above Basal
55 mM KCl	
Normal DPBS	565 $\pm$ 82
Ca^{2+} -free DPBS	0 $\pm$ 0
BD737 (100 μ M)	
Normal DPBS	60.5 $\pm$ 9.47
Ca^{2+} -free DPBS	62.3 $\pm$ 7.33
Red HAL (100 μ M)	
Normal DPBS	40.1 $\pm$ 4.68
Ca^{2+} -free DPBS	28.0 $\pm$ 1.0
CB-64D (100 μ M)	
Normal DPBS	210 $\pm$ 18
Ca^{2+} -free DPBS	220 $\pm$ 30

TABLE 3

Effect of Thapsigargin-Pretreatment on Acute Response to Sigma Ligands

Plated SK-N-SH cells were treated for 10 min with 150 nM thapsigargin (THAP) to deplete Ca^{2+} stores in the endoplasmic reticulum. The $[\text{Ca}^{2+}]_i$ in the cells was monitored to assure a return to a stable baseline. After the return to (or near) the original baseline, 1 mM carbachol or 100 μM sigma ligand was added, and the maximum change in $[\text{Ca}^{2+}]_i$ was calculated as described in the legend to Fig. 2. Values are compared with those in control cells not treated with THAP. Values are expressed as percent change in $[\text{Ca}^{2+}]_i$ relative to the original basal level in nontreated cells or to the new baseline achieved after 10 min of THAP treatment, which was always at or near the original basal level. The effect of thapsigargin on carbachol-induced rise in $[\text{Ca}^{2+}]_i$ was investigated as a control for effectiveness of the thapsigargin treatment. Carbachol (1 mM) value in nontreated cells is the average of two cells $\pm$ S.E.M. and value in THAP-treated cells is the average of four cells $\pm$ S.E.M. The sigma ligands BD737, reduced haloperidol, and CB-64D were used at a concentration of 100 μM , and values are the averages of three cells $\pm$ S.E.M. for nontreated and three to four cells $\pm$ S.E.M. for THAP-pretreated. Experiments with carbachol and BD737 were carried out in normal DPBS, whereas reduced haloperidol and CB-64D were examined in Ca^{2+} -free DPBS.

Condition	% Increase in $[\text{Ca}^{2+}]_i$ above Basal
Carbachol (1 mM)	1,968 $\pm$ 306
THAP/Carbachol	87.5 $\pm$ 22.4
BD737 (100 μM)	60.5 $\pm$ 6.14
THAP/BD737	5.5 $\pm$ 4.8
Red HAL (100 μM)	28.0 $\pm$ 1.0
THAP/Red HAL	0 $\pm$ 0
CB-64D (100 μM)	220 $\pm$ 30
THAP/CB-64D	0 $\pm$ 0

cells. The rise in $[\text{Ca}^{2+}]_i$ was extremely rapid, and the return to baseline was steeper than that observed with sigma-2 ligands. The carbachol-induced rise in $[\text{Ca}^{2+}]_i$ reached peak level in ~ 2 to 4 s and declined to a level baseline in ~ 60 s after reaching the peak (profile not shown). Pretreatment of cells with 150 nM thapsigargin almost completely blocked this robust carbachol-induced rise in $[\text{Ca}^{2+}]_i$, confirming that the conditions that were used effectively deplete the endoplasmic reticulum Ca^{2+} store.

The effect of thapsigargin on the action of sigma ligands is shown as representative time-versus- $[\text{Ca}^{2+}]_i$ traces in Fig. 5. The addition of thapsigargin (150 nM) caused a robust transient rise in $[\text{Ca}^{2+}]_i$, which is indicative of intracellular store depletion. In all cells examined, the thapsigargin-induced rise in $[\text{Ca}^{2+}]_i$ began immediately on the addition of thapsigargin and returned to near baseline levels within ~ 10 to 13 min. The subsequent addition of 100 μM BD737 or CB-64D produced no rise in $[\text{Ca}^{2+}]_i$. Thus, thapsigargin treatment eliminated the normal effect of the sigma ligands demonstrated in Fig. 1, suggesting that sigma ligands release Ca^{2+} from the endoplasmic reticulum. Table 3 gives the averaged results from several cells and shows that the thapsigargin pretreatment blocked the rise in $[\text{Ca}^{2+}]_i$ produced by the sigma ligands BD737, Red HAL, and CB-64D (100 μM). Furthermore, the data in Table 3 and Fig. 5 show that extracellular Ca^{2+} had no effect on either the thapsigargin-induced rise in $[\text{Ca}^{2+}]_i$ or the ability of thapsigargin pretreatment to block the effect of sigma ligands, because similar results were obtained in the presence and absence of extracellular Ca^{2+} .

The converse experiment gave similar results. SK-N-SH cells in normal DPBS were first treated with 100 μM Red HAL, BD737, or CB-64D for 10 to 15 min and then 150 nM thapsigargin after the return to baseline. Data were compared with cells treated with thapsigargin alone. The thapsigargin-induced rise in $[\text{Ca}^{2+}]_i$ was reduced from $175 \pm 6.7\%$

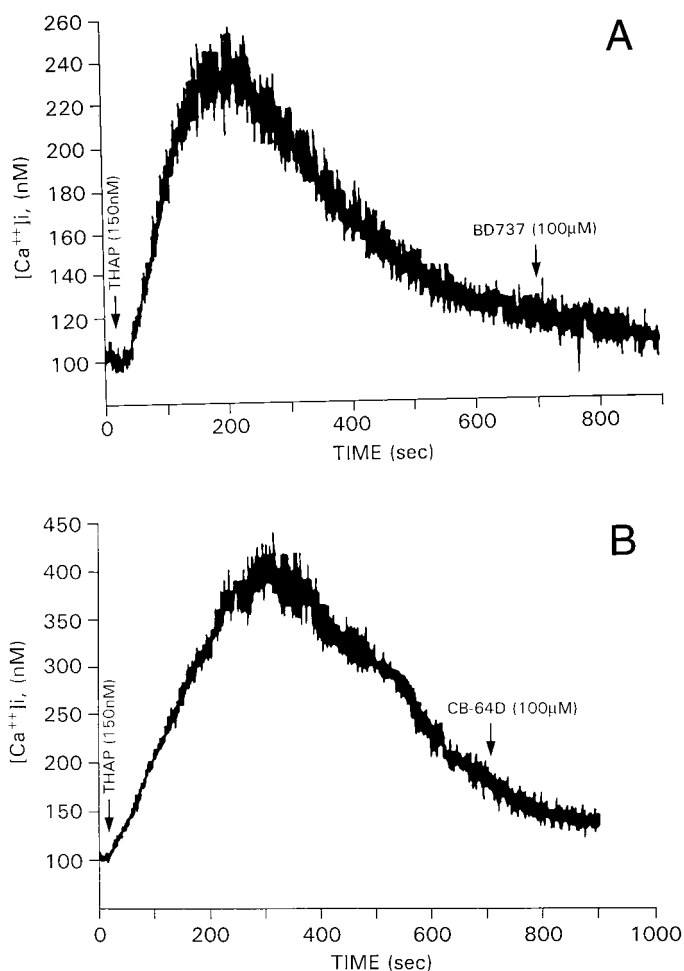


Fig. 5. Time-versus- $[\text{Ca}^{2+}]_i$ traces showing effect of thapsigargin pretreatment on ability of sigma ligands to transiently increase $[\text{Ca}^{2+}]_i$. Thapsigargin (150 nM) was added to cells in either normal DPBS or Ca^{2+} -free DPBS at the indicated time point, and the effect on $[\text{Ca}^{2+}]_i$ was monitored as described in *Materials and Methods* and the legend to Fig. 1. Representative time-versus- $[\text{Ca}^{2+}]_i$ traces are shown. Thapsigargin produced a transient rise in $[\text{Ca}^{2+}]_i$ that began within seconds after the addition and returned to baseline within 10 to 13 min, indicative of depletion of Ca^{2+} from the endoplasmic reticulum and subsequent removal from cell. At the peak, the thapsigargin-induced rise in $[\text{Ca}^{2+}]_i$ was $155 \pm 9\%$ ($n = 58$ cells) relative to the initial basal level. After the return to baseline, 100 μM BD737 (A) or CB-64D (B) was then added as indicated by the arrow, and $[\text{Ca}^{2+}]_i$ was continually monitored. The effect of thapsigargin on BD737 was carried out in normal DPBS, whereas the effect on CB-64D was carried out in Ca^{2+} -free DPBS. No effect on $[\text{Ca}^{2+}]_i$ was seen after the addition of sigma ligand to thapsigargin-treated cells.

($n = 41$ cells) above basal to $65.3 \pm 12.3\%$ ($n = 3$ cells), $15.0 \pm 9.2\%$ ($n = 3$ cells), and $23.0 \pm 1.8\%$ ($n = 4$ cells) above basal when thapsigargin was applied 10 to 15 min after 100 μM Red HAL, BD737, and CB-64D, respectively. This again indicates that thapsigargin and sigma ligands release Ca^{2+} from the same pool.

As mentioned, the Ca^{2+} release profile produced by carbachol was very different in both magnitude and time course compared with that of sigma-2 ligands. This indicates a significant difference in the mechanism of Ca^{2+} gating produced by IP_3 receptors and sigma-2 receptors. In contrast, the profile of Ca^{2+} release produced by thapsigargin resembled that produced by sigma-2 ligands. Although the rising and falling phase of the Ca^{2+} transient tended to be somewhat faster for sigma-2 ligands, there was similarity in both magnitude and

duration (compare Figs. 1 and 5). To address any relationship of sigma-2 ligand-induced release of Ca^{2+} and thapsigargin-induced release, the effect of a sigma antagonist on the action of thapsigargin was investigated. As described above, the experiment was carried out at pH 8.2. The percent rise in $[\text{Ca}^{2+}]_i$ produced by 150 nM thapsigargin alone and by thapsigargin in the presence of 30 μM BD1063 (added simultaneously) was $281 \pm 24\%$ ($n = 3$) and $316 \pm 15\%$ ($n = 4$), respectively. Thus, sigma antagonists had no effect on the ability of thapsigargin to release Ca^{2+} . Taken together, these data show that sigma ligands release Ca^{2+} from the same thapsigargin-sensitive pool as agonists coupled to phosphoinositide turnover (i.e., the endoplasmic reticulum). Furthermore, release by sigma ligands appears to be by a mechanism distinct from that of either thapsigargin or IP_3 .

Effect of Prolonged Exposure to Sigma Ligands on $[\text{Ca}^{2+}]_i$

Effect of Selective Sigma Ligands and Pharmacological Profile. The transient rise in $[\text{Ca}^{2+}]_i$ produced by sigma ligands as characterized above occurred within seconds of the addition of the compound, and in most cells, it had returned to near baseline within ~ 10 min. Observation of the cells by phase contrast microscopy at the time of maximum rise in $[\text{Ca}^{2+}]_i$ or shortly after the return to near baseline showed no sign of morphological changes. Because longer-term exposure of cells to sigma ligands produces changes in morphology (Bowen and Vilner, 1994; Vilner et al., 1995a), we investigated the effect of prolonged exposure on $[\text{Ca}^{2+}]_i$.

The longer-term effects of sigma ligands on $[\text{Ca}^{2+}]_i$ were determined either in normal DPBS or in Ca^{2+} -free DPBS by leaving the cells in contact with sigma ligand for a prolonged period after monitoring the initial rise and return to baseline. After a stable baseline was obtained, $[\text{Ca}^{2+}]_i$ was determined by taking readings at 10-min intervals, with cells being exposed to light only during Ca^{2+} measurement to minimize bleaching of the Indo-1 dye. A typical result from a cell exposed to 100 μM CB-64D is shown in Fig. 6. As shown earlier, CB-64D produced an almost immediate, rapid, and transient rise in $[\text{Ca}^{2+}]_i$, with a return to near baseline in ~ 2 min in this particular experiment. However, within 12 min after the return to baseline, a gradual and sustained rise in $[\text{Ca}^{2+}]_i$ was observed to begin. This rise in $[\text{Ca}^{2+}]_i$ continued for up to 60 min. Observation of the cells in phase contrast mode over this time period revealed changes in morphology such as shortening or loss of processes and beginning phases of cell rounding.

The pharmacological profile of this latent, sustained rise in $[\text{Ca}^{2+}]_i$ was identical to that observed for the immediate, transient rise. Compounds tested at 100 μM were CB-64D, BD737, Red HAL, DTG, and (+)-pentazocine (see legend of Fig. 6). CB-64D, BD737, and Red HAL were active, with all producing the latent, sustained rise in $[\text{Ca}^{2+}]_i$. However, (+)-pentazocine produced no rise in $[\text{Ca}^{2+}]_i$ above basal in up to 60 min of exposure. This again suggests mediation by sigma-2 receptors. Furthermore, as observed for the transient effect, the anomalous inactivity of DTG was also observed here.

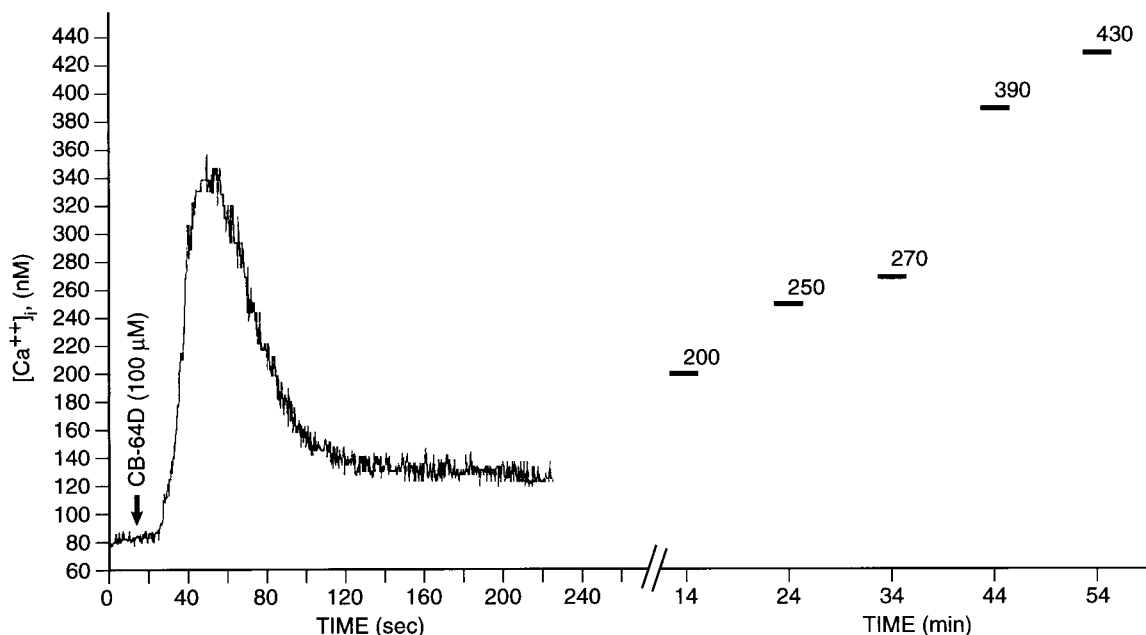


Fig. 6. Effect of prolonged exposure of SK-N-SH neuroblastoma cells to sigma ligand. The sigma ligand was added to SK-N-SH neuroblastoma cells at the point indicated, and the $[\text{Ca}^{2+}]_i$ was monitored continuously for ≥ 5 min or until Ca^{2+} levels returned to near original baseline. The shutter was then closed. After ~ 15 min had elapsed since drug addition, the $[\text{Ca}^{2+}]_i$ was determined every 10 min for up to 70 min by taking readings over a few seconds. This diminished bleaching of Indo-1 dye due to prolonged exposure to light. Cell movement and/or premature detachment often hindered observation of cells for prolonged periods. This was particularly problematic in Ca^{2+} -free DPBS, where cells are attached less strongly than in normal DPBS. This was remedied by growing the cells on coverslips in collagen gel instead of on dry collagen as described in *Materials and Methods*. Alternatively, cells grown on dry collagen could be stabilized by placement under a small piece of glass wool after selection in phase contrast mode. These procedures did not affect the normal response of the cells. Experiments with 100 μM CB-64D were carried out in normal DPBS ($n = 5$ cells) and in Ca^{2+} -free DPBS ($n = 6$ cells) with similar results. Shown is a representative time-versus- $[\text{Ca}^{2+}]_i$ plot for CB-64D (100 μM), carried out in Ca^{2+} -free DPBS. The horizontal bars represent points at which readings were taken, and the $[\text{Ca}^{2+}]_i$ (nM) is displayed beside each bar. Similar results were also obtained with 100 μM BD737 and 100 μM Red HAL (normal DPBS, $n = 3$ cells each). However, neither 100 μM (+)-pentazocine nor 100 μM DTG (normal DPBS, $n = 3$ cells each) had any effect on $[\text{Ca}^{2+}]_i$ for up to 60 min.

Origin of Sustained Increase in $[Ca^{2+}]_i$ Induced by Sigma Ligands. Figure 7 shows a direct comparison of the effect of extracellular Ca^{2+} removal and thapsigargin pretreatment relative to the normal condition on both the transient and sustained rise in $[Ca^{2+}]_i$ produced by CB-64D. The sustained rise, like the transient rise, was observed in the absence of extracellular Ca^{2+} . This shows that the source of the sustained rise in $[Ca^{2+}]_i$ is also intracellular. However, unlike the transient rise, the sustained rise in $[Ca^{2+}]_i$ was not affected by thapsigargin pretreatment, indicating that the intracellular source is not the endoplasmic reticulum. In fact, thapsigargin pretreatment had no effect on the sigma ligand-induced sustained rise in $[Ca^{2+}]_i$ even when extracellular Ca^{2+} was excluded. Taken together, these results suggest that in addition to a rapid and transient release of Ca^{2+} from the endoplasmic reticulum, prolonged exposure of cells to sigma-2 receptor ligands results in a latent and sustained release of Ca^{2+} from other intracellular stores that are thapsigargin-insensitive. This latent release of Ca^{2+} may be associated with alterations in cellular morphology.

Discussion

Here, we further characterized sigma-1 and sigma-2 receptors in human SK-N-SH neuroblastoma cells and demonstrated the presence of sigma-2 sites with properties somewhat distinct from those of rodent tissues (Table 1). It is not clear at the present whether this represents sigma-2 receptor heterogeneity or simple species differences. However, recent studies on the modulation of NMDA receptor function by sigma ligands in rat brain has provided functional evidence indicating possible sigma-2 receptor heterogeneity (Couture and Debonnel, 1998).

Sigma receptor ligands from various structural classes

that include aryloethylene diamine, butyrophenone-related, 5-phenylmorphane, and indole alkaloid produced a dose-dependent, transient rise in $[Ca^{2+}]_i$ in SK-N-SH neuroblastoma cells. Despite a requirement for micromolar concentrations, the effect was highly specific for ligands with sigma-2 binding affinity. Importantly, the action of sigma ligands was blocked by two sigma antagonists, BD1047 and BD1063 (Matsumoto et al., 1995). Thus, the sigma ligand-induced rise in $[Ca^{2+}]_i$ was specifically mediated by sigma-2 receptors, and active ligands are acting as sigma-2 receptor agonists. The only anomaly was the weak activity of DTG, a known sigma agonist with high sigma-2 affinity (Hellewell and Bowen, 1990; Matsumoto et al., 1995).

The removal of extracellular Ca^{2+} had no effect on the percent increase in $[Ca^{2+}]_i$ produced by sigma ligands, whereas pretreatment of the cells with thapsigargin completely eliminated this effect (Tables 2 and 3). The results showed that thapsigargin, carbachol, and sigma ligands all release Ca^{2+} from a common intracellular storage pool: the endoplasmic reticulum (Table 3 and Fig. 5). A comparison of the effect of CB-64D in the presence and absence of extracellular Ca^{2+} (Fig. 7) reveals that although the peak level of $[Ca^{2+}]_i$ reached is the same under both conditions, the return to near baseline occurs more quickly in the Ca^{2+} -free medium. Thus, the broader peak of $[Ca^{2+}]_i$ that occurs in the presence of extracellular Ca^{2+} is actually composed of two components: a component derived solely from the endoplasmic reticulum and a component derived extracellularly. This indicates that the activation of sigma-2 receptors may result in capacitative Ca^{2+} entry, where the release of Ca^{2+} from the endoplasmic reticulum subsequently triggers the opening of Ca^{2+} release-activated channels in the plasma membrane to allow the entry of extracellular Ca^{2+} to replenish the intracellular store (Putney, 1990). The possible

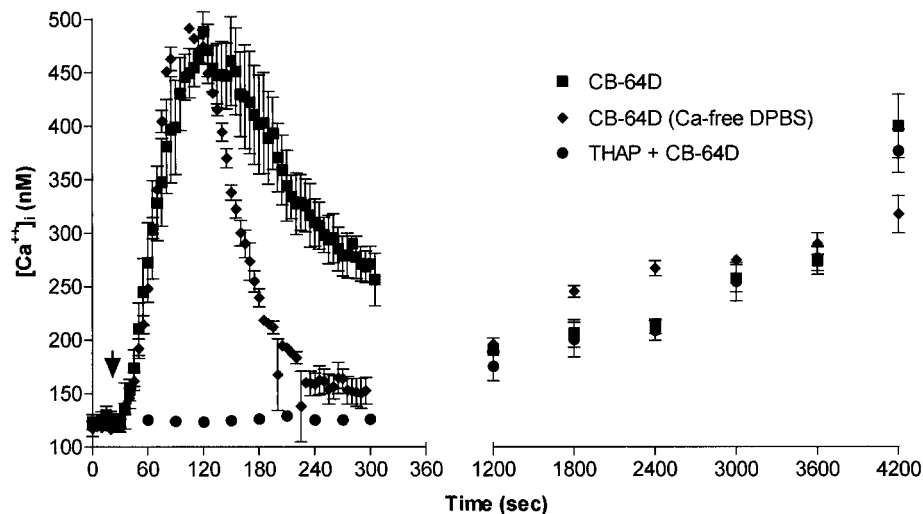


Fig. 7. Effect of thapsigargin pretreatment and removal of extracellular Ca^{2+} on ability of sigma ligands to induce a latent rise in $[Ca^{2+}]_i$. Experiments were carried out essentially as described in the legend of Fig. 6. For the data shown, cells in normal DPBS were treated with vehicle for 10 min (■) or with 150 nM thapsigargin for 10 min (●) before the addition of 100 μ M CB-64D at the time point indicated by the arrow. The effect of removal of extracellular Ca^{2+} (without thapsigargin pretreatment) is included for comparison (◆), with CB-64D added at the point shown by the arrow. Values of the individual points are the averages of three experiments $\pm$ S.E. After producing an initial, transient rise in $[Ca^{2+}]_i$ itself, thapsigargin alone (150 nM) produced no further effect on $[Ca^{2+}]_i$, with Ca^{2+} levels remaining at or near the normal basal level for up to 70 min in the absence of a sigma ligand ($n = 9$ cells in normal DPBS; data not shown). Thapsigargin pretreatment (●) abolished the rapid transient Ca^{2+} signal produced by CB-64D but had no effect on the latent sustained rise in $[Ca^{2+}]_i$. Except for a more rapid return to baseline for the transient rise, removal of extracellular Ca^{2+} (◆) had no effect on the magnitude of either the transient rise or the latent, sustained rise. It should be noted that similar results were obtained in thapsigargin-pretreated cells regardless of whether extracellular Ca^{2+} was present when cells were exposed to CB-64D: thapsigargin experiments with 100 μ M CB-64D were carried out in normal DPBS ($n = 9$ cells) or in Ca^{2+} -free DPBS ($n = 6$ cells) with similar results. Furthermore, thapsigargin pretreatment gave similar results with 100 μ M BD737 and 100 μ M Red HAL ($n = 3$ cells each in normal DPBS; $n = 3$ cells each in Ca^{2+} -free DPBS).

involvement of Ca^{2+} release-activated channels in the actions of sigma-2 receptors in these cells deserves further investigation.

Sigma receptor ligands also induced a sustained rise in $[\text{Ca}^{2+}]_i$ (Figs. 6 and 7). This was apparent only after longer-term exposure of cells to sigma ligands and may account for the incomplete return to baseline observed with the transient phase (Fig. 1). The latent, sustained rise in $[\text{Ca}^{2+}]_i$ persisted in the absence of extracellular Ca^{2+} and in cells pretreated with thapsigargin. Mitochondria are the most likely source for this Ca^{2+} . However, release could also occur from thapsigargin-insensitive Ca^{2+} storage pools known to be present in other organelles such as Golgi apparatus, secretory vesicles, or nucleus (Pozzan et al., 1994; Clapham, 1995; Simpson et al., 1995). The pharmacological profile of the sustained rise in $[\text{Ca}^{2+}]_i$ was identical to that of the transient Ca^{2+} rise. Furthermore, as with the transient rise in $[\text{Ca}^{2+}]_i$, DTG was anomalously inactive. This indicates that these two effects on $[\text{Ca}^{2+}]_i$ are closely related processes. The exact source and mechanism of release require further investigation.

The mechanism by which sigma-2 receptor ligands cause intracellular release of Ca^{2+} from the endoplasmic reticulum in SK-N-SH neuroblastoma cells is not known. It was recently shown that nanomolar concentrations of sigma ligands cause increases in IP_3 formation and modulation of Ca^{2+} transients in adult rat cardiac myocytes (Novakova et al., 1998). However, these effects in cardiac myocytes appear to involve sigma-1 receptors, because (+)-pentazocine produces potent effects (Ela et al., 1994). Furthermore, unlike the case with cardiac cells, sigma ligand-induced stimulation of inositol phosphate production could not be detected in brain synaptoneurosome or in neuronal cell lines (Walker et al., 1990; Bowen et al., 1992, 1993b; Cutts et al., 1993). In fact, sigma ligands [e.g., (+)-pentazocine, DTG, haloperidol, Red HAL, BD737, BD1008] blocked the ability of muscarinic agonists to stimulate phosphoinositide turnover in these systems, including SK-N-SH neuroblastoma cells. It is therefore unlikely that sigma ligands would gate Ca^{2+} via IP_3 production in neuronal cells. However, it is possible that production of low levels of IP_3 went undetected in previous studies, and the effect of selective sigma-2 receptor agonists on phosphoinositide turnover in SK-N-SH neuroblastoma cells will be reinvestigated.

Sigma-2 receptors could potentially influence Ca^{2+} release from the endoplasmic reticulum by influencing the activity of either the IP_3 -gated Ca^{2+} channel (IP_3 receptor) or ryanodine-gated Ca^{2+} channel (ryanodine receptors). Interestingly, sigma-1 receptors were recently shown to potentiate the IP_3 -induced Ca^{2+} release resulting from the stimulation of NG-108 cells with bradykinin, apparently by enhancing the action of IP_3 at IP_3 receptors (Su et al., 1998). Because the activity of IP_3 and ryanodine receptors is susceptible to modulation by effectors such as protein kinases and other endogenous molecules, it is at least conceivable that sigma-2 receptor activation could result in activation of these Ca^{2+} channels by a downstream signaling mechanism in the absence of IP_3 production (IP_3 receptor) or membrane depolarization (ryanodine receptor).

Because sigma-2 ligands and thapsigargin produced similar Ca^{2+} release profiles (compare Figs. 1 and 5), another possibility is that sigma compounds inhibit the SERCA pump. Thapsigargin exhibited no affinity ($K_i > 10 \mu\text{M}$) for either sigma-1 sites or sigma-2 sites (B.J.V. and W.D.B., unpublished observa-

tion), suggesting that currently defined sigma binding sites are not synonymous with a thapsigargin-sensitive Ca^{2+} -ATPase. Furthermore, the effect of sigma-2 receptor ligands was blocked by sigma receptor antagonists, whereas sigma receptor antagonists had no effect on the ability of thapsigargin to release Ca^{2+} . Thus, if SERCA pump inhibition is involved, it is unlikely that sigma-2 ligands and thapsigargin would act via the same mechanism. The data support a receptor-mediated mechanism for the sigma ligands. It is possible, however, that activation of sigma-2 receptors might result in negative modulation of Ca^{2+} -ATPase activity by a downstream signaling mechanism.

Sigma receptors have been localized in high density to subcellular fractions of brain and liver tissue, particularly endoplasmic reticular fractions (McCann and Su, 1990; Basile et al., 1992) and mitochondrial fractions (Itzhak et al., 1991), and in lower density in plasma membrane fractions (McCann and Su, 1990). Furthermore, the cloned sigma-1 receptor has been found to contain an endoplasmic reticulum localization motif at the amino terminus (Hanner et al., 1996). The localization of sigma receptors in subcellular fractions known to store Ca^{2+} could suggest an intracellular site of action for sigma-2 ligands to gate Ca^{2+} .

An intracellular site of action for sigma-2 ligands could explain some apparent inconsistencies in the data. 1) Although the sigma-2 binding affinities for the active ligands are in the nanomolar range, micromolar concentrations are required for effects on $[\text{Ca}^{2+}]_i$. If the site of action is intracellular, the ligands may have difficulty crossing the cell membrane, thus requiring a high extracellular concentration to achieve a nominally effective intracellular concentration. 2) Ligands with similar sigma-2 binding affinity do not always have similar potencies. This may be due to differences in the ability to penetrate the cell membrane. 3) DTG, despite high sigma-2 affinity, is practically inactive. DTG might be expected to show little or no activity because, being a guanidine, it is highly charged at physiological pH and might have great difficulty entering cells. Several recent observations support an intracellular site of action for sigma-2 ligands (Bowen et al., 1999). The potency of sigma-2 ligands at increasing $[\text{Ca}^{2+}]_i$ and inducing cytotoxicity was directly related to the hydrophobicity (LgP value) and was increased when the extracellular pH was raised from 7.2 to 8.2. This suggests that deprotonation and the resultant increased lipid solubility enhance the activity of the compounds. Also, the relatively hydrophilic sigma antagonists BD1047 and BD1063 were effective blockers of ligand-stimulated rises in $[\text{Ca}^{2+}]_i$ at pH 8.2, as shown in Figs. 3 and 4, but were largely inactive at pH 7.2. This is consistent with deprotonation leading to increased intracellular access of the antagonists to the site of agonist action. The greater blockade with antagonist pretreatment (Fig. 4) likely reflects time needed for antagonist to adequately diffuse into the cell.

A rise in $[\text{Ca}^{2+}]_i$ is known to play a central role in cellular toxicity (Berridge et al., 1998). This results from activation of several Ca^{2+} -dependent processes, including Ca^{2+} -dependent proteases and nitric oxide production. Ca^{2+} has also been implicated in triggering apoptosis via activation of Ca^{2+} -dependent endonucleases and other enzymes involved in cell destruction (McConkey and Orrenius, 1996). We have shown that certain compounds that exhibit sigma affinity produce profound morphological changes and cell death in human SK-N-SH neuroblastoma cells, rat C6 glioma cells,

and several other neuronal and non-neuronal cell lines that contain sigma-1 and sigma-2 receptors (Vilner et al., 1995a). Similar effects occur in rat cerebellar granule cells and primary cultures of other areas of rat nervous system (Bowen and Vilner, 1994). We have now demonstrated that the prolonged activation of sigma-2 receptors results in apoptotic death in these cells (Vilner and Bowen, 1997; Vilner et al., 1998). Furthermore, it has been shown that Red HAL induces apoptosis and a prolonged rise in $[Ca^{2+}]_i$ in MCF-7 adenocarcinoma and WIDr colon carcinoma cells (Brent et al., 1996). Therefore, it is feasible that the observed sigma-2 receptor-mediated rises in $[Ca^{2+}]_i$ might be linked in some way to sigma-2 receptor-mediated morphological changes and induction of apoptosis in various kinds of cells. It is also feasible that the transient, sigma ligand-induced rise in $[Ca^{2+}]_i$ might subserve a normal signaling role in the varied modulatory actions observed with sigma ligands, such as neurotransmitter synthesis and release and electrophysiological activity.

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